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Antigen Specific Recognition is Critical for the Function of Regulatory $CD8^{+}CD28^{-}$ T cells

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Deutsche Zusammenfassung

Als Teil des erworbenen Immunsystems spielen T-Zellen eine wichtige Rolle in der Bekämpfung von Krankheitserregern, indem sie körperfremdes Antigen erkennen und so eine Immunantwort verursachen. Allerdings existieren nicht nur T-Zellen, die Immunantworten induzieren und unterhalten, sondern auch solche, die eine Immunreaktion verhindern bzw. beenden. Diese Zellpopulationen sind Gegenstand vieler Forschungsarbeiten, da ihre Anwendung als zelluläres Therapeutikum für Autoimmunerkrankungen oder in der Transplantationsmedizin eine erfolgversprechende Alternative zu nebenwirkungsreichen Pharmaka darstellt.

In dieser Arbeit werden murine $CD4^+CD25^+(T_{reg})$ und $CD8^+CD28^-$ (T_{sup}) T-Zellen untersucht, zwei T-Zell-Populationen mit immunregulatorischer Wirkung. Bisherige Untersuchungen haben gezeigt, dass diese Zellen *in vitro* unter anderem dadurch immunsuppressiv wirken, indem sie die Proliferation von Effektor-T-Zellen unterdrücken. Unklar ist allerdings bis dato, über welchen Mechanismus diese Zellen ihre Funktion ausüben. Da für konventionelle T-Effektor-Zellen die Aktivierung durch den T-Zell Rezeptor unabdingbar ist, stellt sich die Frage, ob T-Zellen mit regulatorischen Eigenschaften auch über diesen aktiviert werden müssen.

Zur Beantwortung dieser Frage wird in der vorliegenden Publikation ein transgenes Mausmodell („DES-Modell“, Hintergrund B10.BR, $H2\text{-}k^k$) genutzt, in dem alle T-Zellen einen Antigen-spezifischen T-Zell Rezeptor (DES-TCR) exprimieren. Zusätzlich exprimieren diese transgenen T-Zellen auch den natürlichen T-Zell-Rezeptor, allerdings in sehr niedriger Dichte. Der transgene T-Zell-Rezeptor erkennt

spezifisch MHC Klasse I Moleküle (in Bindung mit Eigenpeptid) von Mäusen auf H2-k^b Hintergrund (z.B. C57BL/6). Da nur $CD8^+$ T-Zellen durch Antigen aktiviert werden können, das auf MHC Klasse I exprimiert wird, kann man davon ausgehen, dass in diesem Mausmodell $CD8^+$ T-Zellen sehr effektiv durch Antigen-präsentierende Zellen (APC) von C57BL/6 (B6) Mäusen stimuliert werden können, $CD4^+$ Zellen allerdings nicht.

Die Arbeitshypothese dieser Arbeit geht davon aus, dass nicht nur die Aktivierung von T-Effektorzellen von einer erfolgreichen, Antigen-vermittelten TCR-MHC Interaktion abhängig ist, sondern auch die Aktivierung von $CD4^+CD25^+(T_{reg})$ und $CD8^+CD28^-$ (T_{sup}) T-Zellen.

Bevor spezifische Versuche zur Bestätigung oder Verwerfung dieser Hypothese durchgeführt wurden, wurden die einzelnen (immunologisch relevanten) Zellpopulationen der verwendeten Mausstämme charakterisiert. Mittels Durchflusszytometrie wurde sichergestellt, dass alle T-Zellen von DES-Mäusen den transgenen DES-TCR exprimieren (Abbildung 1A). Des Weiteren konnten die relativen Anteile an Immunzellen in der Milz anhand von Oberflächenmarkern bestimmt werden (Abbildung 1B). Hervorzuheben ist der unterschiedliche Anteil von CD4 und CD8 Zellen in DES-Mäusen ($CD3CD4^+$: 7,0%, $CD3CD8^+$: 12,2%) verglichen mit B10.BR Mäusen (CD4: 13,0%, CD8: 8,5%). Die Existenz von $CD4^+$ T-Zellen in der DES Maus ist auf die (in der Literatur beschriebene) Expression des endogenen, natürlichen TCR zurückzuführen, der im Thymus mit MHC Klasse II interagieren kann.

Nach phänotypischer Charakterisierung der Milzzell-Subpopulationen aller verwendeten Mausstämme, wurden $CD4^+$ und $CD8^+$ Zellen von DES Tieren funktionell mit denen des Wildtyp-Stamms (B10.BR) verglichen. Dazu wurden die magnetisch aufgereinigten T-Zellen mit CD90-depletierten Milzzellen für fünf

Tage stimuliert. Anschließend wurde die T-Zell-Proliferation mittels CFSE Verdünnung und die Aktivierung mittels CD25-Expression gemessen. CD8 Lymphozyten von DES Mäusen proliferieren deutlich stärker nach Stimulation mit H2-k^{b+} APC verglichen mit Wildtyp-Zellen, ebenso ist die Dichte von CD25 auf diesen Zellen erhöht (Abbildung 2). $CD4^+$ T-Zellen zeigten hinsichtlich ihres Proliferationsverhaltens keinen signifikanten Unterschied in CFSE und ^{3}[H]-Thymidin-Assays, auch wenn die Proliferation von $CD4^+$ DES Zellen in allen Versuchen niedriger war als die von Wildtyp Zellen (Abbildung 3). Diese Gruppe an Versuchen zeigt, dass der transgene TCR auf DES Zellen eine TCR-MHC vermittelte Aktivierung von T-Zellen ermöglicht, weswegen auch alle DES $CD8^+$ Zellen profilieren, während nur ein Teil der Wildtyp-CD8 Zellen proliferiert.

Da der transgene DES-TCR auf Effektorzellen zu einer Aktivierung führt, stellte sich nun die Frage, ob auch $CD8^+CD28^-$ T-Zellen in ihrer Funktion (und damit antiproliferativen Wirkung) durch erhöhte Antigen-Spezifität verstärkt sind. Dazu wurden $CD4^+$ Wildtyp-Zellen (H2-k^k) mit CD90-depletierten allogenen APC (H2-k^b) stimuliert und mit $CD8^+CD28^-$ T Zellen supprimiert (Abbildung 4). Während T_{sup} von DES-Mäusen in allen erprobten Verdünnungen im CFSE-Assay die Proliferation von $CD4^+$ Zellen nahezu vollständig unterdrücken konnten, zeigten Wildtyp T_{sup} nur ein mäßiges Suppressionspotential. Auch im Tritium-Inkorporationstest war die Aktivität in den DES-Gruppen im Vergleich zur entsprechenden Wildtyp-Kontrolle stark verringert. Der Antigen-spezifische T-Zell-Rezeptor auf DES $CD8^+CD28^-$ Zellen führt also zu einer deutlichen Verstärkung des immunsuppressiven Potentials bei Stimulation mit dem entsprechenden Antigen. Im Vergleich dazu konnten T_{reg} aus DES Tieren in keiner der eingesetzten Verdünnungen die Reaktion von $CD4^+$ Zellen nach Stimulation mit Alloantigen unterdrücken.

Zusammenfassend wird in der vorliegenden Arbeit die Abhängigkeit der Funktion von T-Zellen mit immunsuppressiven Eigenschaften

von einer TCR-MHC Interaktion untersucht. Da der transgene TCR auf DES Mäusen nur mit MHC Klasse I interagieren kann, sind nur $CD8^+$ T-Zellen voll funktional, während $CD4^+$ T-Zellen auf den (viel niedriger exprimierten) endogenen TCR angewiesen sind. Folglich proliferieren $CD8^+$ T-Zellen nach Stimulation mit dem entsprechenden Antigen deutlich stärker als Wildtyp-Zellen. Die Proliferation von $CD4^+$ T-Zellen ist weniger stark ausgeprägt, weil das T-Zell-Rezeptor-Repertoire zu ungunsten der MHC II-abhängigen T Zellen verschoben ist. Antigen-spezifische T_{reg} sind in der Literatur beschrieben, und die in dieser Arbeit aufgeführten Versuche unterstreichen die Wichtigkeit einer intakten TCR-MHC Interaktion, da T_{reg} von DES Mäusen ein deutlich verringertes suppressives Potential aufweisen. Des Weiteren unterstreicht die Arbeit die Antigen-spezifische Wirkung von T_{sup} Zellen, da DES $CD8^+CD28^-$ T-Zellen deutlich stärker immunsuppressiv wirken als ihre Wildtyp-Kontrollen. Weiter zu erforschen gilt es, inwiefern sich die vorliegende Analyse mit Ergebnissen in Einklang bringen lässt, nach welchen nicht-antigenspezifische T_{reg} eine wichtige Rolle bei Autoimmunerkrankungen und in der Tumorimmunologie spielen.

Insgesamt gesehen unterstützt die vorliegende Arbeit das Konzept einer Antigen-spezifischen immunsuppressiven Therapie mit Hilfe von T-Zellen des regulatorischen Phänotyps.

Antigen Specific Recognition is Critical for the Function of Regulatory $CD8^{+}CD28^{-}$ T cells

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Abstract

The immunomodulatory properties of CD8 T cells with regulatory phenotype have become evident. It remains unclear whether the immunomodulatory function of CD8+ CD28- T cells requires antigen-specific TCR interaction with major histocompatibility complex class I (MHC-I). We have isolated naïve CD8+CD28- T suppressor cells (**Tsup**) from H2-Kk Des-TCR mice that express a transgenic, MHC class I-restricted, clonotypic TCR against an allogeneic MHC class I molecule (H2-Kb) plus self-peptide. These cells were compared to B10.BR wildtype (w/t) CD8+CD28- T cells and to naïve CD4+CD25+ regulatory T cells (**Treg**) of the same strains. Des CD8 effector T cells proliferated more readily when stimulated by H2-Kb splenocytes than w/t controls, whereas Des CD4 T cells showed the same alloresponse as w/t cells. Activation and proliferation of B10.BR CD4 T cells stimulated by H2-Kb APC was suppressed more effectively by Des CD8+CD28- T cells than by w/t CD8+CD28- T cells. On the contrary, Des CD4+CD25+ T cells inhibited T cell proliferation less effectively than w/t CD4+CD25+ T cells. In conclusion, we demonstrate that the function of naive Tsup is strongly enhanced by antigen recognition. Therefore we expect that Tsup are possible candidates for antigen-specific immunosuppressive therapy.

Keywords

CD8, CD28, T suppressor cells, T regulatory cells, TCR, MHC class-I, antigen recognition

Introduction

Regulatory T cells with a variety of phenotypes have been described to control the balance between immunity and tolerance in the periphery [1] [2] [3]. Most of these cells belong to the CD4 compartment, like the prototypic CD4+CD25+ T cells (Treg). However, T cells with a regulatory function have also been identified within the CD8 subset [4] [5]. The functional relevance of so-called CD8+CD28- T suppressor cells (Tsup) has been suggested in disease models [6] and clinical scenarios [7] [8]. Their physiological role as well as the exact mechanism by which they down-regulate the immune response, however, remain unclear. Recognition of target antigen by a specific TCR is the central dogma of the adaptive, cell-mediated immune response. Thus, one might expect that not only initiation, but also subsequent down-regulation of the specific immune response by specialized suppressor T cells should be antigen-specific. While it has been described that activation, but not maintenance of CD4+CD25+ T cell function is dependent on antigen recognition [9], it remains controversial whether the same is true for CD8+CD28- suppressor T cells.

CD8+CD28- T suppressor cell subsets with antigen-specific function have been described [10]. These antigen-specific CD8+CD28- T cells inhibit CD4 responses in a MHC class I-restricted manner. Generation of tolerogenic dendritic cells (DC) by antigen-specific CD8+CD28- T cells has been suggested to be the dominant mechanism through which the alloresponse is down-regulated [11]. Tolerized DC, in turn, can then anergize allospecific CD4 cells. Contradicting these findings, other studies have shown that CD8 suppressor cells do not express FoxP3 and do not require antigen recognition but rather suppress the alloresponse in an IFN-γ dependent manner [12].

To determine the role of antigen in Tsup function, we used a well-characterised TCR-transgenic murine system in which antigen-specific Tsup can be isolated [13], and investigated the requirement of antigen-recognition for the function of CD8+CD28- and CD4+CD25+ Des T cells. We demonstrate here that antigen-specific Tsup are more efficient suppressors of T-cell proliferation in presence of their respective antigen when compared to wildtype controls. CD4+CD25+ naive regulatory cells of the same strain that cannot bind antigen through the transgenic receptor are less effective. Thus, we believe that CD8-mediated control of the immune response carries the principal potential for antigen-specific immunosuppression.

Methods

Mice

6-8 week old C57BL/6 mice (H2-Kb haplotype) were purchased from Charles River Laboratories (Wilmington, MA, USA). B10.BR (H2-Kk haplotype) and Des-TCR mice [13] (H2-Kk haplotype, transgenic TCR) were a kind gift of B. Arnold (Heidelberg, Germany). All mice were bred in our institute's animal facility under standard conditions. All animal experimentation was carried out in accordance with the regional authorities under the legislation of the administration of the Upper Palatinate, Germany.

Flow cytometry and antibodies

For flow cytometry analysis, cells were washed and resuspended in phosphate-buffered saline (PBS). Cell suspensions were incubated with monoclonal antibodies at 4°C for 20 minutes with combinations of saturating amounts of PacificBlue™-, fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)-, AlexaFluor™ 700-, PerCP-, PE-Cy5-, allophycocyanin (APC)- and APC-Cy7-conjugated antibodies purchased from Becton Dickinson (Heidelberg, Germany) or eBioscience (San Diego, USA). Clonotypic Désiré antibody [29 30], specific for the transgenic TCR was generated in-house. For the detection of living cells, propidium iodide (Sigma Aldrich, St. Louis, USA) or DAPI was added directly before analysis. Cells were analyzed on a FACS Calibur or LSR-II flow cytometer (Becton Dickinson) using either CellQuest or FACSDiva Software (Becton Dickinson).

Cell Purification

CD4+CD25+ T regulatory cells were isolated and purified from spleen single cell suspensions using a commercial kit (Miltenyi, Bergisch Gladbach, Germany) according to the manufacturer's protocol, or by staining of splenocytes with CD25-PE and CD4-APC antibodies followed by cell sorting with a FACSAria flow cytometry sorter (Becton Dickinson, Heidelberg, Germany).

For purification of CD8+CD28- cells, single cell suspensions of splenocytes were first stained with CD28-PE antibody and subsequently depleted from CD28 positive cells using paramagnetic anti-PE microbeads (Miltenyi, Bergisch Gladbach, Germany). In the next step, cells were positive-selected for CD8+ using anti-CD8-BEADS in the same system (Miltenyi, Bergisch Gladbach, Germany). For mixed lymphocyte cultures, the same technique was applied to purify responder cells with anti-CD4-beads, and to deplete stimulator cells of CD90+ cells with anti-CD90-beads (Miltenyi, Bergisch Gladbach, Germany).

CFSE staining and mixed leukocyte reactions (MLR)

Allogeneic mixed leukocyte reactions were performed with 10^5 magnetically purified CD4+ or CD8+ splenocytes from either B10.BR or Des-TCR donors as responders. To observe proliferation events, responder cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) (Invitrogen, Karlsruhe, Germany) using a protocol described before [14]. In brief, cells were washed twice in FCS-free medium and then incubated with 5 µmol CFSE per 10^6 cells for five minutes at 37°C. The labelling procedure was stopped by adding FCS containing medium. As stimulators, $2x10^5$ irradiated (30Gy), CD90 depleted C57Bl/6 splenocytes were used. Responder

cells stimulated by CD90-depleted syngeneic cells served as controls. MLRs were carried out in 96-well round bottom plates in a total volume of 200µl RPMI (Gibco) cultivation medium (supplemented by 0.1% 50mM 2-Mercaptoethanol, 1% MEM-non-essential Amino-Acids, 2% 1M HEPES (pH 7,36-7,39/ NaOH), 1% 100mM Sodium-Pyruvate solution, 1% 100x Antibiotic/ Antimycotic, 1% MEM Vitamin solution, 1% 200mM L-Glutamine and 10% heat-inactivated FCS) and analyzed by flow cytometry after five days of culture.

For ^{3}[H]thymidine incorporation assays, 0.5 µCi ^{3}[H]thymidine (PerkinElmer, Boston, USA) per well was added 18 hours prior to termination of the co-culture. Cells were harvested onto a microfibre plate (Wallac, Turku, Finland) and radioactivity was measured using a liquid beta scintillation counter (Wallac, Turku, Finland).

Software

Flow cytometry data was computed by FlowJo V7.1.3 software (TreeStar, Inc., Ashland, OR, USA). A students' t-test was used for statistical comparison (GraphPad Software Inc., La Jolla, CA, USA). $p<0.05$ was considered statistically true.

Results

Expression of transgenic T-Cell Receptors in the Des-TCR system

All CD4 and CD8 lymphocytes purified from the Des-TCR mice express the transgenic TCR (Figure 1A). However, due to incomplete allelic exclusion of the TCR alpha chain, a significant subset of Des-TCR T cells expresses two different alpha chains: one being rearranged endogenously and the other representing the product of the transgene [15]. As a result, in addition to the transgenic Des-TCR, some CD8 T cells and all CD4 T cells T cells express an endogenously rearranged TCR of unknown specificity. CD4+ T cells do not develop in Des-TCR RAG-1-/- mice, suggesting that positive selection of these cells occurs via the endogenous TCR. However, the overall number of Des-TCR CD4 cells is decreased (Figure 1B) and these CD4 T cells have an impaired TCR repertoire because of their lower frequency of naturally recombining, CD4-dependent TCR. Furthermore, it has to be considered that Des-TCR CD4 T cells co-express high levels of fully transgenic TCR and lower levels of natural TCR although they do not express the costimulatory machinery to function through MHC class I.

The transgenic Des-TCR CD8 response towards H2-Kb alloantigen is more effective than the w/t response, whereas both transgenic Des-TCR CD4 T cells and B10.BR w/t controls proliferate moderately upon stimulation with H-2Kb alloantigen

To compare the extent of the proliferative response of transgenic versus wild type CD8 cells after stimulation with H-2Kb alloantigen, we stimulated purified Des-TCR and B10.BR CD8 cells with irradiated H-2Kb positive CD90-depleted splenocytes in vitro. The

proliferative response was analyzed by CFSE dilution. Five days after stimulation, all CD8+ transgenic Des-TCR cells had undergone several divisions, whereas only a fraction of wild type cells proliferated upon alloantigen stimulation (Figure 2A). The CD25 expression of transgenic CD8 T cells was significantly higher than that of their wild type counterparts indicating greater activation by alloantigen (Figure 2B).

In contrast to CD8 T cells, proliferation of purified Des-TCR CD4 T cells activated by H2-Kb positive splenocytes did not result in a significantly different response compared to wild type controls in a thymidine incorporation assay (Figure 3A). Data from the radioactive incorporation assay was additionally confirmed by a CFSE dilution assay. Here, several population doublings for Des-TCR and wild type CD4 T cells were observed, although, in this more sophisticated assay, the CD4 Des-TCR response was less pronounced (Figure 3B). In addition, CD25 expression of CD4 Des-TCR cells was decreased compared to their wild type controls (Figure 3C), indicating weaker overall activation.

Transgenic CD8+CD28- Des-TCR T cells suppress responder cell proliferation upon H2-Kb stimulation more effectively than w/t CD8+CD28- T cells

After having outlined that both CD4 and CD8 T cells of Des-TCR and wild type origin proliferate upon TCR stimulation by alloantigen, whereby the response of transgenic CD8 cells was markedly increased, we further investigated whether the regulatory potential of the transgenic cell population was also stronger than that of their wild type counterpart. For this purpose CD8+CD28- T cells of Des-TCR and wild type origin were purified and tested for their ability to

inhibit the proliferation of allogeneic CFSE-labeled B10.BR CD4 responder T cells responding to H2-Kb+ stimulators.

In this MLR, CD8+CD28- suppressor T cells suppressed CD25 up-regulation and proliferation of CD4 responder cells independently of their origin. Interestingly, wild type CD8+CD28- T cells suppressed responder cell proliferation in a CFSE dilution assay significantly less than Des-TCR CD8+CD28- cells and never reached baseline levels (Figure 4A). The effect of transgenic cells was more pronounced and not dose dependent in the chosen fractions, indicating a considerable higher suppressive activity of this TCR-transgenic population (Figure 4B). This observation was confirmed in a thymidine incorporation assay (Figure 4C). Here, radioactive thymidine uptake was clearly higher when wildtype Tsup were used. In addition a decrease in CD25 up-regulation of responding CD4 cells could be outlined (data not shown).

In contrast to TCR transgenic Des-TCR CD8+CD28- cells, CD4+CD25+ Des-TCR cells do not suppress the CD4 alloresponse versus H2-Kb

To further follow our hypothesis that interaction between the transgenic Des-TCR and H2-Kb governs the regulatory function of CD8 cells, we isolated Des-TCR and wild type CD4+CD25+ T cells. These DES CD4+CD25+ T cells cannot be fully activated by antigen through the transgenic TCR because CD4 cells lack the costimulatory machinery to function in a MHC class I-restricted fashion. Thus, if CD4+CD25+ T cells regulate the alloresponse through antigen recognition, DES cells can only function through their endogenously rearranged TCR, which is less frequent on DES T cells. In a corresponding MLR, B10.BR CD4+ responder cells strongly proliferate when stimulated with allogeneic H2Kb+ splenocytes. And

indeed, this proliferation can be significantly suppressed by wild type CD4+CD25+ regulatory T cells, whereas Des-TCR CD4+CD25+ T cells completely fail to suppress this proliferation (Figure 5). Thus, our transgenic mouse model provides further evidence that adequate TCR signaling is also critical for the suppressive function of naïve CD4 T regs.

Discussion

Antigen recognition through specific TCR is the key to adaptive T-cell immunity. It remains an open debate whether down regulation of the immune response by T cells with regulatory or suppressor phenotype in the immunological periphery is also exclusively antigen-dependent. In our current paper we used a transgenic mouse strain (Des-TCR mice) that expresses clonotypic TCR against murine MHC class I (H2-Kb) plus self peptide. The transgenic TCR is expressed on all T cells and is highly MHC I-dependent. Thus, it is only fully functional on CD8+ T cells. Des-TCR CD4 cells expressing the transgene escape thymic selection of Des-TCR mice since they co-express lower densities of naturally recombined TCR. Although the Des-TCR receptor is dominant on these CD4 cells, the naturally recombining receptor is present to a lower extent. This initial disadvantage of the Des-TCR system was used in the present model to isolate Des-TCR positive CD4+CD25+ cells as an intrinsic control[16]. Thus, our murine experimental system is suited to compare the function of CD4 and CD8 T-cell populations with regulatory and suppressor phenotype in an antigen-restricted transgenic setting (compare table 1).

Our results are consistent with a model in which peripheral T-cell immunity is mediated best by T cells that are highly specific for their target antigen. And indeed, TCR-transgenic Des-TCR CD8 cells mount a significantly higher proliferative response when stimulated with H2-Kb antigen than their non-transgenic, wild type counterparts under the same conditions of stimulation. This finding is well in keeping with the observation that CD8 cells with a narrow TCR repertoire (as it is provided through a transgenic receptor) respond most readily to foreign antigen, processed from e.g. virus, allograft or tumor [17] [18] [19]. On the other hand, even transgenic Des-

TCR CD4 cells responded to H2-Kb antigen stimulation in our model, indicating that Des-TCR CD4 cells are capable of performing their effector function by endogenous receptor, albeit showing less distinctive proliferation compared to controls.

Naïve CD4+CD25+FoxP3+ regulatory T cells are the most widely studied regulatory T cell population. These cells were clearly outlined to suppress allograft rejection and GVHD in vivo [20] [21]. At present, clinical protocols are initiated to introduce Tregs into clinical transplantation medicine [22]. For the case of these non-specifically expanded CD4+CD25+ regulatory T cells it has become evident, that only a fraction of the population actively suppresses the targeted immune response in an antigen-specific manner. Thus, it will be desirable to isolate antigen-specific Tregs to prevent general immunosuppression through transplanted Tregs [23] [24] [25]. For CD4+CD25+ Tregs, it has been shown that activation requires antigen recognition [9]. Our observation is in keeping with this finding since Des-TCR CD4+CD25+ T cells did not express a regulatory function due to the dominant presence of transgenic TCR on Des-TCR Tregs. Thus, it may be assumed that activation of CD4+CD25+ through the TCR and the respective costimulatory molecules is necessary also in our model.

Unlike CD4+CD25+ Tregs, CD8+CD28- T suppressor cells may harbor the capability of acting very efficiently in a non-antigen-restricted fashion. Thus, impaired function of non-antigen-specific Tsup has been discussed for autoimmune disorders [12] and non-antigen-specific Tsup were also identified in human cancers [26]. Furthermore, failure to generate Tsup from the periphery is a feature of HIV infection [27]. In contrast to these well-documented observations, results from our model outline that highly antigen-restricted CD8+CD28- Des-TCR Tsup are significantly stronger suppressors of CD4 responder cell proliferation than wild type controls. Thus, our findings underline the importance of an antigen-dependent TCR-

MHC class I interaction for the immunosuppressive function of Tsup, as an increased frequency of antigen-specific Tsup leads to an increased anti-proliferative effect, at least under the present conditions. Other authors have discussed the functional relevance of Tsup that mediate a tolerogenic DC phenotype in an alloantigen-specific manner [28]. These features of antigen-restricted Tsup also seem to play an important role in the maintenance of transplantation tolerance [10].

Collectively, we show that CD8+CD28- T suppressor cells are antigen-dependent in vitro in a transgenic model of CD8-mediated immunity. Indirectly, CD4+CD25+ regulatory T cells were also confirmed to be antigen-dependent. This finding suggests a potential of T cells with regulatory phenotype for tailored immunosuppression in vivo.

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Figure Legends

Figure 1

(A) Single cell suspensions were analyzed by flow cytometry. Splenocytes from Des-TCR donors were gated on living CD3+ leukocytes. Both CD3+CD4+ and CD3+CD8+ lymphocytes stained positive for Des-TCR detected by antibody Désiré. Controls were assessed but not shown for figure clarity. (B) B10.BR, Des-TCR and C57Bl/6 splenocytes were characterized for major leukocyte populations. Notably, the frequency of Des-TCR CD3+CD4+ T cells was decreased as compared to wild type controls, whereas CD3+CD8+ T cells were more frequent in Des-TCR mice. Mean values of three independent experiments are shown.

Figure 2

(A) DES and wild type B10.BR CD8 lymphocytes were stimulated by CD90-depleted C57BL/6 (H2-Kb) splenocytes. DES CD8+ T cells (black) proliferated more effectively upon this allogeneic stimulation as compared to wild type controls (grey). (B) These findings were confirmed by an increase of CD25 expression on DES CD8 T cells. All experiments were carried out in triplicates. Representative experiments are shown.

Figure 3

(A) CD4+ B10.BR or DES splenocytes were co-cultured with irradiated CD90 negative H2-Kb splenocytes for five days. 3[H]thymidine incorporation reveals an increased proliferation of

both DES and B10.BR CD4 lymphocytes as compared to syngenically-stimulated controls (*). However, proliferation of DES CD4 cells is not less effective when compared to wild type CD4 cells (n.s.). Error bars illustrate SD. (B) The same setting was used for a CFSE dilution assay. Here, wild type CD4+ cells (grey) showed a slightly higher proliferation than DES CD4+ cells (black). Dotted and dashed lines represent syngenically-stimulated controls. (C) CD25 expression of wild type CD4 was higher than that of DES CD4 lymphocytes after stimulation. All experiments were carried out at least three times. Representative plots are shown.

Figure 4

(A) Wild type B10.BR CD8 Tsup suppressed CD4 T cell proliferation only slightly in a CFSE dilution assay. A higher Responder:Tsup dilution resulted in minor suppression. (B) In contrast, all chosen dilutions of DES Tsup strongly suppressed proliferation of CD4 lymphocytes to baseline levels. (C) Proliferation of B10.BR CD4 T cells was also suppressed by different numbers of wild type or transgenic CD8 Tsup in a thymidine assay. Here, DES Tsup inhibited the proliferation of CD4 cells more effectively than wild type Tsup., although no difference reached statistical significance when compared to allogeneic stimulated controls. All experiments were carried out at least three times. Representative plots are shown.

Figure 5

Different numbers of wild type or transgenic CD4+CD25+ Treg were used to suppress CD4 T cell proliferation of B10.BR donors. Wild type Treg were suppressive depending on the number of Treg cells administered, whereas Treg from DES donors did not decrease the uptake of 3[H]thymidine. (*) $p<0.05$ or (n.s.) $p>0.05$ indicate statistical significance between allogenically stimulated controls and groups suppressed by Tregs. The experiment was carried out three times, a representative plot is shown.

Table 1

	B10.BR (w/t)	Des-TCR
CD4 effector function	+++	++
CD8 effector function	+	+++
Treg function	++	o
Tsup function	+	+++

Figure 1

fig. 1

B

	B10.BR		DES		C57BL/6	
	Mean	SEM	Mean	SEM	Mean	SEM
NK cells	2.5	0.3	2.1	0.2	3.4	0.2
B cells	60.0	2.4	53.5	2.2	55.0	0.9
DCs	1.0	0.3	0.9	0.2	0.7	0.2
Macrophages	2.3	0.9	4.8	0.8	1.1	0.5
CD4 T cells	13.0	1.1	7.0	0.4	17.4	1.1
CD8 T cells	8.5	0.7	12.2	1.2	11.4	1.6

Figure 2

fig. 2

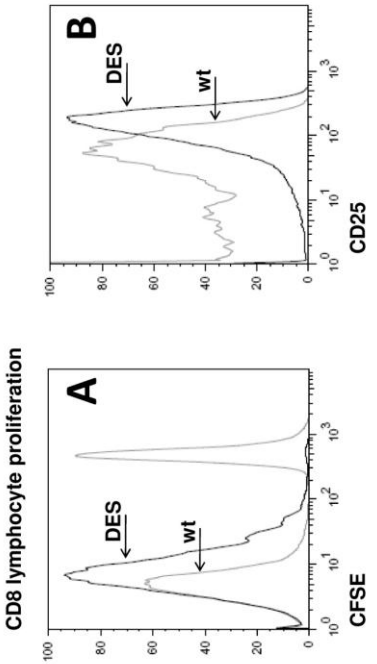


Figure 3

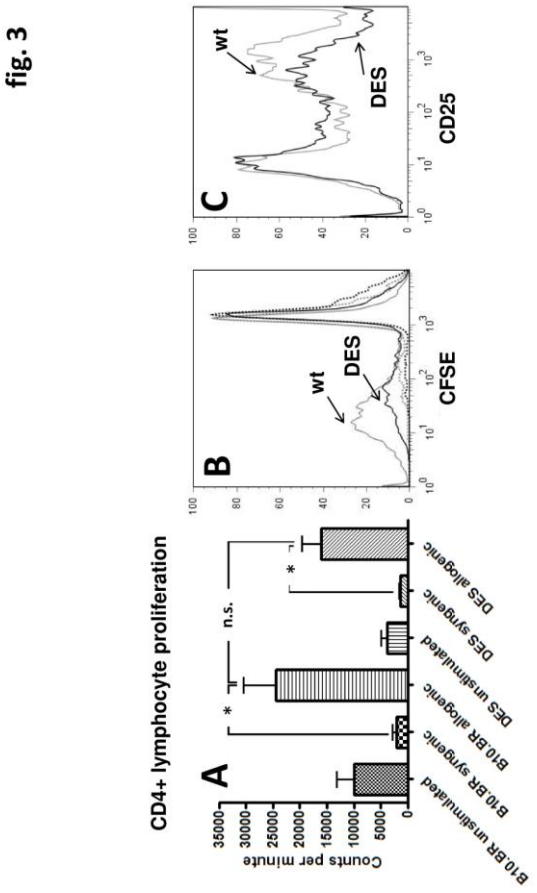


fig. 3
CD4+ lymphocyte proliferation
A
Counts per minute
35000
30000
25000
20000
15000
10000
5000
0
*
n.s.
*
B10.BR unstimulated
B10.BR syngenic
B10.BR allogenic
DES unstimulated
DES syngenic
DES allogenic
B
wt
DES
CFSE
C
wt
DES
CD25

Figure 4

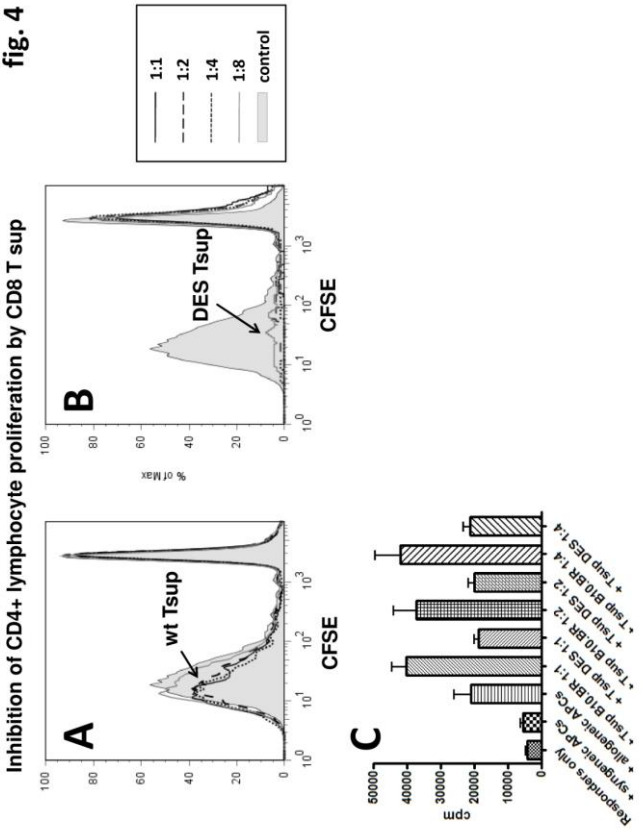
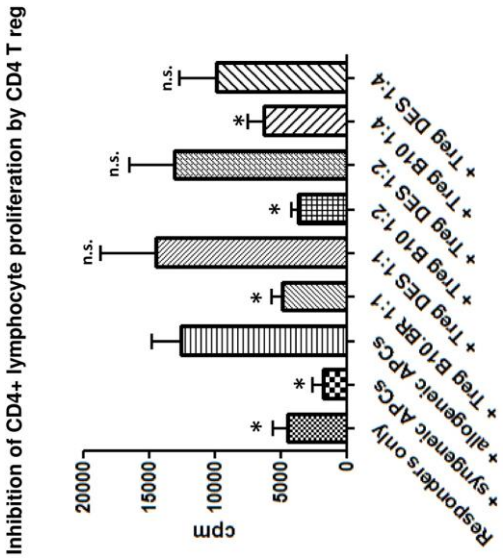


fig. 4
Inhibition of CD4+ lymphocyte proliferation by CD8 T sup
A
wt Tsup
CFSE
B
DES Tsup
CFSE
% of Max
1:1
1:2
1:4
1:8
control
C
cpm
Responders only
+ syngeneic APCs
+ allogeneic APCs
+ Tsup B10.BR 1:1
+ Tsup DES 1:1
+ Tsup B10.BR 1:2
+ Tsup DES 1:2
+ Tsup B10.BR 1:4
+ Tsup DES 1:4

Figure 5

fig. 5



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Regensburg im Januar 2011

Erklärungen

ZUM
ANTRAG AUF ZULASSUNG ZUR
PROMOTION ALS DOKTOR DER MEDIZIN

vorgelegt von
Philipp Renner

unter der Betreuung von
PD Dr. med. Marc-H. Dahlke, PhD

Nach § 6 Absatz 5 der Promotionsordnung der Universität Regensburg vom 12.06.2008:

Ich erkläre, dass ich die der Universität Regensburg zur Promotion eingereichte Dissertation mit dem Titel

Antigen specific recognition is critical for the function of regulatory CD8+CD28− T cells

in der Klinik für Chirurgie der Universitat Regensburg unter der Betreuung von PD Dr. med. Marc-H. Dahlke, PhD ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe.

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